Workshop to Identify Critical Windows of Exposure for Children's Health: Reproductive Health in Children and Adolescents Work Group Summary

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This work group report addresses the central question: What are the critical windows during development (preconception through puberty) when exposure to xenobiotics may have the greatest adverse impact on subsequent reproductive health? The reproductive system develops in stages, with sex-specific organogenesis occurring prenatally and further maturational events occurring in the perinatal period and at puberty. Complex endocrine signals as well as other regulatory factors (genetics, growth factors) are involved at all stages. Evidence from animal models and human studies indicates that many specific events can be perturbed by a variety of toxicants, with endocrine-mediated mechanisms being the more widely studied. Prioritized research needs include basic studies on the cellular–molecular and endocrine regulation of sexual differentiation and development; increased efforts regarding potential adverse effects on development in females, including breast development; expanded animal studies on different classes of chemicals, comparing responses during development (prenatal and postnatal) with responses in adults; and, more extensive explorations regarding the reproductive biology and toxicology of puberty in humans. *Key words*: children, fetal, gametes, gonads, reproduction, sexual development, urogenital system. — *Environ Health Perspect* 108(suppl 3):505–509 (2000).
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This report summarizes issues discussed by a work group on reproductive effects on children's health. Critical periods during growth and development were discussed with respect to the central question: What are the windows of time during development (preconception through pregnancy, childhood, and puberty) when exposure to xenobiotics may have the greatest adverse impact on subsequent reproductive health? A background paper prepared for the work group provided a review of key events in the development of the reproductive system in humans and test species and served as a starting point for discussions concerning critical windows of exposure with respect to subsequent reproductive health (*1*). Development is a broad topic that encompasses the entire life cycle. Therefore, the work group first agreed that a working definition of the term critical window provided in the background paper would be adopted and used to focus discussion on only those health outcomes that involve differentiation, development, or adult functioning of the reproductive system. The definition is repeated here:

> Critical windows of development are limited temporal intervals characterized by the occurrence of sets of dynamic organizational events that constitute periods during which exposures may have the greatest potential to affect later reproductive competency. (*1*)

The work group determined that an event or process would define the window rather than a specific time period, as the latter varies greatly in duration among species. The intent of this report is to capture the issues raised in the discussion and summarize the work group's recommendations for further research. Selected examples from the toxicology and epidemiology literature are used to illustrate the points raised, with the recognition that this report is not intended to be a comprehensive review of the literature.

Background

Relative to many other species, humans exhibit a high rate of infertility, inadequate fecundity, and other reproductive disorders. The extent to which this poor performance may be related to exposure to environmental pollution is of great interest to regulatory agencies. Relevant to this workshop is whether the developing or maturing organism may be differentially sensitive to reproductive toxicants at critical periods of development. It is difficult to address this question due to the high background of infertility and reproductive health problems in the U.S. population. Based on recently released data from the 1995 National Survey of Family Growth, approximately 1 in 10 women in the United States is infertile (*2*). While the causes of infertility can be identified in some cases (such as blockage of ducts that transport gametes as may arise

secondary to medical conditions), others are poorly understood (such as ovulatory disorders) or of entirely unknown etiology (*3*). Furthermore, the incidence of abnormal pregnancy outcomes such as preterm deliveries and low birth weight offspring is also high and appears to be increasing. For example, the percent of babies born preterm, i.e. at less than 37 completed weeks of gestation, increased from 9.4% in 1981 to 11.0% in 1996, and the percent of low birth weight infants increased by 10% from 6.7% in 1984 to 7.4% in 1996 (*4*). These increases may be related to demographic factors that influence the risk of having a premature birth or low birth weight infant, such as attempting pregnancy at an older age or having multifetal pregnancies secondary to the use of ovulation-inducing hormone therapy (*3,5,6*). However, toxic substances released into the environment in recent years may also be a factor. The challenge is to detect, against this background, human reproductive risk attributable to environmental factors encountered during development.

The complexity of the reproductive system also makes it difficult to identify reproductive risks possibly due to exposures early in life or during fetal development. The reproductive system is immature at birth, with considerable differentiation occurring during puberty. While morphologic landmarks of puberty such as Tanner scales in humans and pubertal indices in rodent test species are useful in identifying gross effects on sexual development (*7,8*), reproductive function (fertility and fecundity) cannot be assessed until after sexual maturation. In humans, this is possible only for the subset of individuals who attempt

pregnancy. Consequently, adverse reproductive effects arising from developmental or childhood exposures may go unnoticed. For example, if a gestational exposure resulted in cervical inadequacy, this effect would not be detectable until adulthood, and then only if the woman became pregnant but could not maintain the pregnancy. Thus, by the time a reproductive disorder is diagnosed, a lengthy period of time may have elapsed, making it difficult to identify a causative or initiating agent or pinpoint the critical window(s) within which the exposure may have occurred.

In addition to these developmental considerations, it is also important to recognize that gender-specific differences in reproductive organs and physiology may result in gender-specific responses to a given toxicant. In short, one sex may be more or less sensitive to a particular toxicant than the other. All these factors complicate reproductive hazard identification and risk assessment.

The background paper (1) identified important stages of development that define windows of susceptibility to reproductive toxicants: the preconception period, the period of gonadal differentiation, the period of urogenital system development, the early postnatal period, and the peripubertal and pubertal periods. Discussion of each of these stages led to the identification of a variety of issues important for children's reproductive health.

The Preconceptional Window

The preconceptional window is the period of time prior to conception when the developing gametes may be susceptible to genetic or epigenetic damage that could affect sexual development after fertilization. We know that damage to spermatozoal DNA can be transmitted to the zygote and may result in a variety of developmental problems from early embryo death to fetal malformations to subtle changes in gene expression (9). Indeed, the final stages of spermiogenesis may be relatively more vulnerable than earlier stages, since the DNA in condensed spermatids is incapable of being repaired. If damage occurs in genes that regulate gonadal determination or differentiation or the endocrine control of reproduction (such as hormone receptor genes), subsequent reproductive potential could be affected. However, mutagens studied to date do not appear to act in such a specific manner.

Likewise, exposures that alter gametic sex chromosomes can have profound effects on reproductive development and subsequent fertility. For example, if exposures during meiosis increase the risk of aneuploidy (wrong number of sex chromosomes) or structural aberrations of the sex chromosomes (such as translocations or deletions) in either the oocyte or the spermatozoon, and the

abnormal gametes participate in fertilization, then the resulting zygote is at risk of abnormal sexual development. The sex chromosomes appear to be more susceptible to nondisjunction than most autosomes (10). Except for XYY (which can arise from YY disomy in the spermatozoon and produces a normal sexual phenotype) and Y0 (which can arise from X nullisomy and is incompatible with life), other numerical errors of the sex chromosomes (such as X0, XXY, XXX) result in abnormal gonadal development and/or abnormal meiotic disjunction and often confer infertility in adulthood (10).

Recent development of chromosome-specific probes has made it possible to identify aneuploidy and translocations involving sex chromosomes in sperm, and epidemiology studies using these methods are being conducted to address the extent to which toxicants may contribute to the risk of sex chromosome (and autosomal) abnormalities (11–14). As chromosome-specific probes become available for rodents (or other species), this approach can be applied in chemical-specific toxicology studies (15,16) and extended to zygotes or preimplantation embryos to examine exposure-related effects on either the sperm or the oocyte chromosomes (17). Advances in molecular biology are also making it possible to identify deletions in the Y chromosome that account for some cases of human infertility (18). The frequency of Y chromosome microdeletions in azoospermic and severely oligozoospermic men is estimated to be about 15%, but such deletions may also occur in infertile men with apparently normal semen. Many of these are thought to arise de novo, and the extent to which they may be induced by environmental contaminants is currently unknown. This subject is ripe for research.

The Prenatal Window for Reproductive Development

Gonadal Differentiation

Early in organogenesis the primordial germ cells, which arise in the yolk sac, proliferate and migrate into the genital ridge and form the indifferent gonad. If this migratory process were impeded, the ultimate gametogenic potential of the gonad might be affected (19). The indifferent gonad then develops into an ovary or testis based on the expression of specific genes (1), a process that can be disrupted by mutations in these genes or alterations in factors that regulate their expression. Continued proliferation of stem cells during this time is critical for establishing a suitable primordial germ cell pool of oogonia or spermatogonia. Also, there is evidence that genomic imprinting may occur in germ cells during this window, thus determining the

subsequent parental allele-specific expression of imprinted genes. Exposure to chemicals that affect DNA methylation (e.g., 5-azacytidine, altered methyl status) may disturb this imprint. There is currently little, if any, experimental data addressing the possibility that exposure to toxicants during this critical window can alter gonadal differentiation.

Urogenital System Development

Once the gonad has differentiated, it produces hormonal signals that regulate urogenital tract development. Under the direction of testosterone and Müllerian inhibiting substance (MIS) produced by the fetal testis, the Wolffian ducts differentiate into the male reproductive tract organs and the Müllerian ducts degenerate. In the absence testosterone, the Müllerian ducts differentiate into the female reproductive tract and the Wolffian ducts regress (20). The regulation of sexual differentiation is complex. For example, in the female, coordination of both proliferation and apoptosis is essential to form a patent female tract. In the male, fusion of the urogenital sinus is required for penis formation, and androgenic stimulation is necessary for descent of the testes through the inguinal canal and into the scrotum. Exposure to chemicals with endocrine activity, such as estrogen mimics, progestins, androgens or antiandrogens, during the critical period for development can induce genital malformations such as hypospadias or testicular maldescent in male rodents. Inappropriate endocrine signals can also masculinize the external genitalia in female rodents (19,21–23). Importantly, such effects would be permanent.

Due in part to the prevalence of similar effects in wildlife, this topic is currently the subject of intense investigation in numerous laboratories worldwide under the umbrella of endocrine disruptors research (24). Under federal mandates, screening tests for chemicals that may act by mimicking hormones through a variety of mechanisms are being developed and validated for use in testing (25). Numerous workshops have been held on this topic in recent years, yielding widespread consensus that a) basic research is needed regarding the hormonal control of sexual differentiation during development (and later) and b) better methods and quantitative models are needed to evaluate effects of compounds with endocrine activity, especially after exposures during critical windows of organogenesis (26,27).

Evidence for homologous effects in humans is also emerging. The classic example is the widely studied estrogenic drug diethylstilbestrol (DES). When given to pregnant women, DES produced a variety of reproductive effects in their offspring, most of which

were not apparent until after puberty [for example, structural abnormalities of the cervix, uterus, and fallopian tubes, and increased risk for adenocarcinoma of the vagina in daughters (22)]. Inappropriate exposure to progesterone may also produce abnormal sexual differentiation. For example, a recent study reported a 5-fold increase in hypospadias in the male offspring of women receiving progesterone subsequent to *in vitro* fertilization (IVF) (28). Women who had IVF received progesterone during early gestation, which may have contributed to the perturbation of the fetal endocrine system. It is also possible that this birth defect in the male offspring may have been related to other factors associated with parental subfertility. In a prospective study of approximately 3,600 male infants in West Germany (29), however, there was no significant increase in hypospadias for those women having exposure to progestins via hormonal pregnancy tests. Dosages and timing of administration were considerably different in these two studies, which could have resulted in these discrepant findings. Clearly, more human studies are needed to examine the potential of hormone mimics in the environment to alter reproductive tract development.

Breast Development

The first stage of breast development occurs during organogenesis and involves differentiation of mammary buds and rudimentary lactiferous ducts in both males and females. Exposure of embryo/fetal mammary tissue to a balanced hormonal environment during this time may be important with respect to the number of estrogen receptors formed and therefore to the future capability of the breast tissue to respond to estrogen. Thus, this period is also a critical window for exposures that alter hormone release or action (1).

The Postnatal to Peripubertal Window for Reproductive Development

Early Postnatal Events

The first six months after birth in the human and the first three weeks of postnatal life in the rodent is another potential critical window for testis development. During this time there is a burst of Sertoli cell proliferation followed by its cessation. This phenomenon establishes the number of Sertoli cells in the adult testis, which in turn determines the number of spermatogonia that can be supported, and hence the limits of sperm production in the adult. Thyroid hormone is thought to regulate Sertoli cell proliferation. In rats, induction of hypothyroidism in the perinatal period (during lactation) delays the cessation of Sertoli cell proliferation. This

results in higher than normal numbers of Sertoli cells and consequently in significantly larger testes that produce increased numbers of sperm after puberty. The same effect is observed when rats are exposed to certain polychlorinated biphenyls (PCBs) during this period, presumably due to the antithyroid activity of PCBs (30). Interestingly, these rats also have enlarged prostates in adulthood. Recent evidence indicates that altered prolactin levels during early postnatal life or during puberty may predispose male rats to prostatic inflammation (31). Similarly, exposure to the pesticide atrazine during this critical period alters prolactin levels and induces prostatic inflammation. These animal studies raise questions about the relative susceptibility of humans to such effects and whether animal models for prostatic inflammation can be used to predict risks for human prostatic disease (benign prostatic hyperplasia or prostate cancer) later in life.

In female rats, inappropriate exposure to androgens during the neonatal period is also detrimental. Such exposures at the critical time give rise to adults with delayed pubertal onset, decreased regularity of ovarian cyclicity, ovaries with diminished numbers of follicles, and cessation of ovulation at an earlier age (19,21).

Peripuberty (Late Childhood) and Puberty (Adolescence)

In response to complex hormonal signals at puberty, the gonads begin to function and produce gametes. This involves the initiation of spermatogenesis from spermatogonia in the testis and the initiation of folliculogenesis from primordial follicles in the female. Recent reviews are available regarding the regulation of puberty in the male and female rat, and these summarize the rat toxicology literature regarding known effects of pharmaceuticals as well as environmental contaminants on puberty and subsequent reproductive function (32,33). These reviews propose test protocols to screen chemicals specifically for endocrine-disrupting activity during this critical window of development. However, little concerted effort has been made to evaluate the relative sensitivity of the gonads during the period of time when gametogenesis is becoming established, compared with the fully functional adult gonad, to chemicals that target the ovary or testis specifically.

The hypothalamic–pituitary–gonadal axis may be an important target for disruption, as it plays critical roles in the establishment of spermatogenesis and ovarian cyclicity during puberty that carry over into adulthood. As is well known in humans, individual deviations from the norm in patterns of steroid production are associated with low fecundability. Until recently, however, little was known about the potential effects of more subtle

changes in hormonal patterns with respect to conception and implantation (34). Subtle changes may be more likely to be associated with effects of environmental exposures (35). For example, in men, mean concentrations of luteinizing hormone showed a significant change during a randomized trial of inhalation exposures to 50 ppm of toluene (36). Heavy metals, solvents, and pesticides with neuroendocrine activity are particularly suspect for their potential to accelerate or delay puberty. However, little human data are available on this subject.

In some cases, developing testes may be more sensitive than adult testes to compounds that target the testis directly (as opposed to working through the neuroendocrine axis). Although data are limited, the susceptibility appears to depend upon a number of factors, including the cellular target(s) and metabolism of the toxicant. For example, immature and pubertal rats appear to be more sensitive than adults to testicular toxicity induced by exposure to phthalate esters, an effect that may be related to age-specific differences in the absorption and/or metabolism of these compounds (37–39). Immature rats are also more sensitive than adults to the pesticide, 1,2-dibromo-3-chloropropane (DBCP) (40), and there is limited evidence that the fetal rat testis may be more sensitive than the pubertal rat testis (41). DBCP was banned after reports of infertility in occupationally exposed men. On the other hand, immature animals are not always more susceptible. For example, in the rat fetal Leydig cells are less sensitive than adult Leydig cells to a well-known Leydig cell toxicant, ethane dimethanesulfonate (42). Prepubertal rats are also relatively insensitive to the testicular toxicity of 1,3-dinitrobenzene, a putative Sertoli cell toxicant, and young adults are less sensitive than older adults (43). In these studies, *in vitro* systems for culture of isolated testicular cells or seminiferous tubule sections are proving to be useful for determining the modes and mechanisms of age-specific differences in toxicity.

Less is known about age-specific sensitivity of females to ovarian toxicants, in part because relatively few ovarian toxicants have been well characterized. In animal studies, 4-vinylcyclohexene diepoxide (VCD) destroys oocytes contained in small preantral follicles. There is some evidence that adult rats may be less susceptible to VCD-induced ovotoxicity than immature rats (44). Depletion of oocytes has been associated with early menopause in women, raising concern about exposures to ovotoxic chemicals, especially given contemporary secular trends toward delaying childbearing. Earlier menopause is also of concern due to its association with other health risks such as osteoporosis and possibly cardiovascular

events. In perimenopausal women, smoking or being exposed to second-hand smoke has been associated with higher levels of follicle-stimulating hormones, possibly implying that exposed individuals may experience earlier menopause (45). Rodent models are not ideal for studying these associations, since depletion of small follicles does not necessarily result in accelerated reproductive senescence in rodents. Rather, rodents appear to have an endogenous feedback or compensatory means of censusing the number of small follicles and adjusting or slowing the rate of future follicular atresia. The female primate may be a better model in this regard. Nevertheless, the rodent studies have shown that complete destruction of oocytes in primordial follicles appears to be associated with the development of ovarian tumors (46).

Another critical window for breast development (and subsequent susceptibility to cancer) appears to overlap the prepubertal and pubertal periods. In rodents, prepubertal exposure to phytoestrogens was shown to protect the mammary gland from carcinogen-induced malignant transformation, possibly by increasing differentiation of the mammary epithelium (47). On the other hand, exposure to the phytoestrogen genistein (but not zearalenone) during pregnancy increased mammary tumorigenesis in the offspring (48). Clearly, endocrine-mediated effects are complex and a systematic examination of the relative sensitivity of the prenatal, prepubertal and pubertal periods is needed.

Comparability of Human and Laboratory Studies

It is important to understand when animal models can be representative of the human reproductive exposure–response and when animal models may be less appropriate. Similarities between other species and the human reproductive systems include a) reproductive tissue architecture and regulation of gametogenesis, b) steroidogenic pathways (with minor differences), c) specific gene expression that directs reproductive development and function, and d) similarities in the nature, if not the magnitude, of effects of specific reproductive toxicants. Although the rodent models are not perfect, rats and mice are the preferred species for reproductive and developmental toxicity testing required by federal guidelines, and there is a large toxicology database in rodents that is used for risk assessment. However, limitations with respect to children's health studies include the relatively short interval (only a few days in absolute terms) between birth and the initiation of gametogenesis, as opposed to years in humans. This short interval in rodents limits interpretation of studies on chemicals that are thought to bioaccummulate in children and makes it

difficult to use rodents to address questions of aggregate or intermittent exposures during childhood in humans. For such studies, it is important to explore the suitability of other animal models such as rabbits and primates that have a longer period of postnatal development before the onset of puberty.

Relevant for interspecies extrapolation with respect to prenatal exposures is the need to understand differences between placentation in rodents and humans and the role of the rodent yolk sac early in pregnancy. These may result in important species-specific differences in xenobiotic availability and metabolism. Finally, when examining effects arising subsequent to prenatal exposures in rodents, consideration should be given to the incidence of abnormalities both within and between litters and, in some cases, to the intrauterine position of the fetus.

Concluding Remarks and Recommendations

Evaluating the impact of xenobiotics on the development and adult functioning of the reproductive system in humans poses some unique challenges. These challenges include a) the intermittent expression and use of the reproductive organs, b) the potential extended delays from the time of initial exposure to awareness that there may be a problem such as infertility, and c) the need for the interaction of two individuals before there is an awareness that a problem exists, e.g., infertility. The use of animal models can overcome some of these difficulties and inform human studies with respect to potential modes and mechanisms of toxicity.

Based on the work group's discussions of existing knowledge about reproductive physiology and about critical windows for exposure to reproductive toxicants, consensus was reached by the reproductive work group on a number of high priority goals. This list is intended to stimulate research to support the risk assessment process at it pertains to reproductive health of children.

- Determine when an exposure is more likely to cause an adverse effect, either qualitative or quantitative, on the developing offspring compared to the mature adult.
- Determine the internal regulatory mechanisms that are set early in development and affect responses later in life.
- Identify the gaps in our understanding of the differences in reproductive development between rodents and humans and determine the relative importance of these differences for risk assessment.
- Evaluate the sensitivity of the primordial follicles to toxicant exposure. Does sensitivity vary in different physiologic states (e.g., pre- vs postnatal, or when there are

bursts of atresia occurring naturally)? In males, are stem cells more or less sensitive before or after birth and before or after puberty?

- Initiate research on female reproduction beyond adverse pregnancy outcomes. For example, a) What is the relationship between external versus internal breast development? b) What is the relationship between serum hormones and anovulation? c) What is the relative sensitivity of immature versus mature ovaries to chemicals that affect folliculogensis?
- Determine when the peripuberty period begins in humans. How sensitive is the peripuberty period to xenobiotics? What organizational events are essential for moving toward puberty?
- Determine to what extent the period of late childhood is sensitive to xenobiotics. For example, peripubertal boys appear to be just as sensitive as adults to chemotherapeutics. More information is needed to address this question for other classes of toxicants.
- In humans, evaluate the relationship of Tanner scales (external markers of puberty) with internal organ development (e.g., appearance of sperm in ejaculate, maturation of the vas deferens, serum hormones).
- Use databases such as the National Health and Nutrition Survey (NHANES) to compare Tanner stages over time as a means of surveillance for environmental effects on puberty in humans. NHANES data might help identify potential childhood developmental risks and contribute to the design and prioritization of future studies.
- Generate more information about exposures of pregnant women and children, especially to endocrine-active agents, including environmental contaminants, drugs, and nutritional supplements, and the potential consequences of such exposures.
- Assess the relative vulnerability of the male and female germ cell to toxicants and evaluate the consequences on subsequent generations.

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